Identification and characterization of the chicken transferrin receptor

Jerzy A. SCHMIDT,*† John MARSHALL‡ and Michael J. HAYMAN‡

Imperial Cancer Research Fund Laboratories, St. Bartholomew's Hospital, Dominion House, Bartholomew Close, London EC1A 7BE, U.K.

Among a panel of monoclonal antibodies that recognize chicken haematopoietic-differentiation antigens, one, JS 8, was found to immunoprecipitate a 95000- M_r cell-surface protein from chicken erythroblasts transformed with avian-erythroblastosis virus. This protein was shown, by affinity chromatography on immobilized chicken transferrin (conalbumin), to be the chicken transferrin receptor. Although immunologically unrelated to the human transferrin receptor, biochemical comparison of the chicken transferrin receptor to the human receptor showed similarities with respect to the pattern of biosynthesis, degree of glycosylation, dimerization in the absence of reducing agents and subcellular localization. The present report contrasts with recent ones describing the chicken transferrin receptor isolated from embryonic tissues as a 58000- M_r protein.

INTRODUCTION

Iron is essential for the growth of all vertebrate cells and is taken up by cells bound to the carrier protein, transferrin (Aisen & Listowski, 1980; Newman *et al.*, 1982). Receptors for transferrin are found on the surface of many eukaryotic cells and, with the exception of reticulocytes, expression of the protein on the cell surface is related to the proliferative state of the cells, with higher surface expression on more rapidly proliferating cells (Larrick & Cresswell, 1979a; Newman *et al.*, 1982). However, during erythropoiesis, iron is additionally required in high amounts for haemoglobin synthesis, and consequently erythroblasts and reticulocytes express high levels of the transferrin receptor irrespective of their proliferative state (Iacopetta *et al.*, 1982).

We have been studying chick erythroid differentiation, in particular cell-surface molecules whose regulation is altered during terminal differentiation, induced *in vitro*, of erythroblasts transformed by temperature-sensitive mutants of avian-erythroblastosis virus (ts AEV) (Beug *et al.*, 1982; Hayman *et al.*, 1982; Adkins *et al.*, 1985). Because of its known role in erythropoiesis and cell proliferation in mammalian cells, we reasoned that the chicken transferrin receptor may be playing an important role in the differentiation of chicken erythroblasts to red blood cells. Therefore we decided to try and identify the chicken transferrin receptor in these cells and to compare its structure with that of mammalian transferrin receptor.

Human and mouse transferrin receptors have been extensively studied. They have been purified from human placenta, reticulocytes, kidney and various mouse and human cell lines (Hu & Aisen, 1978; Larrick & Cresswell, 1979b; Seligman *et al.*, 1979; Fernandez-Pol & Klos, 1980; Faulk *et al.*, 1980; Trowbridge & Omary, 1981) and have been shown to be transmembrane glycoproteins of

M_r 180000–190000 that are composed of two identical subunits in a disulphide-linked dimer. Recent studies with monoclonal antibodies against the receptor have allowed its characterization in more detail (Omary & Trowbridge, 1981; Schneider *et al.*, 1982), and these antibodies immediately proved to be useful probes in functional studies of the molecule (Trowbridge & Lopez, 1982). Also, cDNA cloning has provided the primary structure of the human and murine receptors (Schneider *et al.*, 1983, 1984; Stearne *et al.*, 1985).

However, despite these similarities in structure, it was found that few antibodies raised against the human transferrin receptor recognized the murine receptor and vice versa. Therefore it was not surprising when preliminary experiments showed that monoclonal (OKT9) and rabbit polyclonal antibodies against human transferrin receptor did not recognize any protein in avian erythroblasts or reticulocytes (J. A. Schmidt, J. Marshall & M. J. Hayman, unpublished work). This meant that the available antibodies could not be used to identify the chicken transferrin receptor. We therefore screened a number of monoclonal antibodies we had raised against antigens regulated during chick erythroid differentiation (Hayman *et al.*, 1982; Schmidt *et al.*, 1985b) and noticed that one, JS 8, preferentially recognized proliferating cells in chicken bone marrow and that the expression of this antigen was increased on transformation of various different avian haematopoietic cells. Therefore the antigen recognized by JS 8 was an obvious candidate for the chicken transferrin receptor by analogy to the expression of mammalian transferrin receptor. In the present paper we show that the protein recognized by JS 8 is indeed the chicken transferrin receptor, and structural studies demonstrate it to have similarities with the mammalian receptor.

Abbreviations used: (ts) AEV, (temperature-sensitive mutant of) avian-erythroblastosis virus; DMEM, Dulbecco-modified Eagle medium; SDS, sodium dodecyl sulphate; NP40, Nonidet P40; FCS, foetal-calf serum.

* Too whom all correspondence and reprint requests should be addressed.

† Present address: Department of Experimental Immunobiology, The Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, U.K.

‡ Present address: Department of Microbiology, State University of New York, Stony Brook, NY 11794, U.S.A.

MATERIALS AND METHODS

Cells

The chicken erythroblast-cell line, HD3, transformed with a temperature-sensitive mutant (*ts34*) of AEV was cultured as previously described (Beug *et al.*, 1982).

Antibodies and lectins

Monoclonal antibodies JS 5 and JS 8 were selected from a fusion of the NS₁ mouse myeloma-cell line with spleen cells from mice immunized with plasma membranes from HD3 cells induced to differentiate *in vitro* as described by Beug *et al.* (1982). Their full derivation is described elsewhere (Schmidt *et al.*, 1985b). Affinity-purified anti-conalbumin immunoglobulin was kindly given by Dr. H. Beug, European Molecular Biology Laboratory, Germany. Rhodamine-conjugated swine anti-rabbit immunoglobulin was purchased from Dako, and fluorescein-conjugated goat anti-mouse immunoglobulin and Rhodamine-conjugated wheat-germ agglutinin were from Sigma.

Radiolabelling of cells

Cells were starved for 15 min in methionine-free DMEM and labelled for 2 h at 35 °C with L-[³⁵S]-methionine (> 30TBq/mmol; Amersham International) at 500 μ Ci/10⁶ cells in 0.5 ml of methionine-free medium. Pulse-chase experiments involved a 5 min pulse with L-[³⁵S]methionine as described above in 0.5 ml of methionine-free DMEM followed by addition of 5 ml of normal growth medium.

Immunoprecipitation

Immunoprecipitation was carried out essentially as described by Schmidt *et al.* (1985a), with the following modifications. It was found that SDS and sodium deoxycholate destroyed antibody binding by monoclonal antibody JS 8, therefore these two detergents were omitted and an incomplete RIPA buffer [10 mM-Tris/HCl (pH 8.0)/1% (v/v) Triton X-100/0.15 M-NaCl/0.02% NaN₃/1 mM-PMSF] was used to form immune complexes. Complete RIPA buffer [containing additional 0.1% (w/v) SDS and 1% (w/v) sodium deoxycholate] was, however, used to wash immunoprecipitates once formed. Monoclonal antibodies were used as a tissue-culture supernatant, 200 μ l/immunoprecipitation, and pre-mixed with an equal volume of double-strength incomplete RIPA before use. Antibody JS 8 is of class IgG1 and thus not bound by protein A, hence rabbit anti-mouse immunoglobulin (0.5 μ l/immunoprecipitation) was used as a bridge between heat-fixed *Staphylococcus aureus* and immune complexes.

For immunoprecipitation from live cells, HD3 cells [(1–2) $\times$ 10⁶] were radiolabelled as described above, washed three times at 4 °C in complete growth medium and incubated with 200 μ l of monoclonal-antibody tissue-culture supernatant on ice for 1 h. Cells were washed a further three times with cold growth medium, lysed in incomplete RIPA (1 ml) and clarified by centrifugation at 12000 *g* for 5 min. Immune complexes were collected by using rabbit anti-mouse immunoglobulin-coated *S. aureus* as described above.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed on 1 mm-thick gels as described by Laemmli (1970).

After running, gels were processed for fluorography as described by Bonner & Laskey (1974), dried and exposed to Kodak X AR5 film.

Affinity chromatography

Immobilization of chicken transferrin (conalbumin; Type II; Sigma Chemical Co.) and bovine serum albumin (Sigma, Type V) to CNBr-activated Sepharose 4B (Pharmacia) and subsequent affinity chromatography to purify chicken transferrin receptor was performed essentially as described for human transferrin receptor by Hunt *et al.* (1984), with modification of the elution procedure. Instead of mild acid elution, the column was eluted sequentially with 50 mM-sodium citrate, pH 5.0, 0.5% (v/v) NP40 (5 column volumes), 50 mM-Tris/HCl, pH 7.4, 0.5% (v/v) NP40 (5 column volumes) and finally 100 mM-acetic acid, pH 3/0.15 M-NaCl (5 column volumes). Fractions were neutralized with 0.1 M-NaOH as required and prepared for immunoprecipitation by addition of an equal volume of double-strength incomplete RIPA buffer.

Immunofluorescence

Double immunofluorescence on fixed and permeabilized erythroblasts with mouse and rabbit antibodies was performed as described by Beug *et al.* (1979). Briefly, washed and permeabilized fixed cells were incubated in monoclonal-antibody tissue-culture supernatant (50 μ l) mixed with rabbit anti-conalbumin immunoglobulin (1:100 dilution of an 8 mg/ml stock solution) at room temperature for 30 min. Control experiments consisted of NS₁ spent culture supernatant mixed with normal rabbit immunoglobulin at the same dilution. Cells were washed four times with phosphate-buffered saline [20 mM-sodium phosphate (pH 7.4)/0.15 M-NaCl] and incubated in a mixture (25 μ l) of Rhodamine-conjugated swine anti-rabbit immunoglobulin [Dako; 1:40 in phosphate-buffered saline+1% (v/v) FCS] and fluorescein-conjugated goat anti-mouse immunoglobulin (Sigma; 1:30 in phosphate-buffered saline+1% FCS) for 30 min at room temperature. After being washed a further four times in phosphate-buffered saline, cells were mounted in a small volume of Mowiol 4 $\times$ 88 as described by Beug *et al.* (1979). Slides were examined on a Leitz Dialux 22 EB fluorescence microscope fitted with the appropriate filters for fluorescein and Rhodamine fluorescence. Pilot experiments were performed by adding primary and secondary antibodies separately to ensure no cross-reactivity of antibody reactions.

RESULTS

Identification of the chicken transferrin receptor

Monoclonal antibody JS 8 is one of a panel of monoclonal antibodies isolated from a fusion of NS₁ mouse myeloma cells and spleen cells from mice immunized with membranes of the *ts34*-AEV-transformed erythroblast cell line HD3 (Schmidt *et al.*, 1985b). This antibody was found to bind to the surface of mainly erythroblasts and reticulocytes in normal chick bone marrow, but viral transformation of cells of the lymphoid, myeloid and erythroid lineages led to increased expression of the antigen (Schmidt *et al.*, 1985b). This prompted us to investigate further whether this antigen was in fact the chicken transferrin receptor.

The epitope recognized by monoclonal antibody JS 8

Fig. 1. Autoradiograph of immunoprecipitates from L-[³⁵S]-methionine-labelled HD3 cells with monoclonal antibody JS 8

Cells were labelled with L-[³⁵S]methionine for 2 h, and JS 8 was used for immunoprecipitation from either live cells (c, e) or whole cell lysates (d, f). NS₁ myeloma-cell culture supernatant was used for control immunoprecipitations from both live cells (a) or whole-cell lysates (b). Samples were subject to SDS/polyacrylamide-gel electrophoresis in the presence (a, b, c, d) or absence (e, f) of 2-mercaptoethanol. ¹⁴C-labelled M_r markers were obtained from Amersham International and contained myosin (M_r 200000), phosphorylase b (M_r 92500), bovine serum albumin (M_r 68000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 30000) and lysozyme (M_r 14400).

was shown by immunoprecipitation from L-[³⁵S]methionine-labelled detergent extracts of HD3 cells to reside on two proteins that migrated with M_r values of 90000 and 95000 on SDS/polyacrylamide gels (Fig. 1, lane d). In the absence of mercaptoethanol, an additional protein of M_r ~200000 was seen, suggesting that at least a fraction of the protein exists as a disulphide-linked dimer in the non-reduced state (Fig. 1, lane f). As mentioned above, antibodies against the human transferrin receptor also immunoprecipitate two proteins of approx. 85000 and 90000 M_r from total cell lysates, which migrate at 180000 M_r in the absence of mercaptoethanol. In this case, the higher- M_r (90000) band is the form of the protein found on the cell surface (Schneider *et al.*, 1982). To investigate whether this is also the case with the protein immunoprecipitated with JS 8, live cell immunoprecipitations were performed. For this, cells were allowed to react with antibody on ice before lysis, so that immunocomplexes were formed only with exposed cell-surface molecules. Excess antibody was washed away, cells lysed and preformed immune complexes

collected with protein A (*S. aureus*) in the usual way. Such live cell immunoprecipitations showed that only the 95000- M_r protein was expressed on the cell surface (Fig. 1, lane c), and this was also at least partly present on the cell surface as a disulphide-linked dimer (Fig. 1, lane e).

Therefore these properties are very similar to those of human transferrin receptor (Schneider *et al.*, 1982), and they suggested to us that the protein recognized by JS 8 could be the chicken counterpart. To test this directly, we partially purified chicken transferrin receptor by using a one-step method from total cell lysates by affinity chromatography on conalbumin (chicken transferrin)-Sephacrose 4B affinity matrix and asked whether monoclonal antibody JS 8 would recognize this protein. The protocol used to elute transferrin receptor from the affinity column (see the Materials and methods section) utilized the known biology of transferrin-transferrin-receptor interactions and was intended to be more specific than the mild-acid or urea elution procedures employed by others (Hunt *et al.*, 1984; Markelonis *et al.*, 1985). We reasoned that pre-elution of the affinity columns with pH 5.0 buffer would mimic events in pre-lysosomal compartments and dissociate iron from the transferrin (Ciechanover *et al.*, 1983). Subsequent elution with pH 7.5 buffer should then release the receptor from the column in a manner similar to the release of apo-transferrin from cells (Ciechanover *et al.*, 1983). Fig. 2 shows that the 95000- M_r protein recognized by monoclonal antibody JS 8 is only found in the fraction eluted from conalbumin-Sephacrose 4B with pH 7.5 buffer. Furthermore, the 95000- M_r protein was not bound and eluted from a control bovine serum albumin-Sephacrose column. Therefore the data indicate that the antigen recognized by JS 8 is indeed the chicken transferrin receptor.

The 90000- M_r protein is a precursor of the 95000- M_r protein

Pulse-chase experiments with the human transferrin receptor were used by others to resolve the relationship between the 85000- and 90000- M_r proteins precipitated from total cell lysates. It was found that the 85000- M_r protein was the primary translation product, and this was subsequently modified into the 90000- M_r form found on the cell surface (Schneider *et al.*, 1982). Similar pulse-chase experiments were therefore used to investigate a precursor-product relationship between the two proteins immunoprecipitated by JS 8. A 5 min pulse label with L-[³⁵S]methionine shows that the chick transferrin receptor is first synthesized as the 90000- M_r form (Fig. 3). Chase experiments show that conversion into the 95000- M_r form begins after a further 15 min and is virtually complete by 30 min (Fig. 3). A similar pattern was seen in the biosynthesis of human transferrin receptor in foetal liver and the Nalm-6 cell line, although the kinetics in this case were slower, with full conversion taking about 2 h (Schneider *et al.*, 1982).

The chicken transferrin receptor contains N-linked carbohydrate

The precursor-product relationship with the human transferrin receptor described above has been shown to be due to processing of attached N-linked carbohydrate chains (Schneider *et al.*, 1982). To investigate whether the chicken receptor is similarly modified with N-linked glycans, we treated HD3 cells with tunicamycin (Sigma Chemical Co.; 5 μ g/ml), an inhibitor of N-linked

Fig. 2. Partial purification of the 95000- M_r protein recognized by JS 8 on a conalbumin-Sepharose 4B affinity matrix

Total cell lysates of HD3 cells labelled with L-[35 S]methionine were subject to affinity chromatography on conalbumin-Sepharose 4B (a) or bovine serum albumin-Sepharose 4B (b) as described in the Materials and methods section. Material bound to both columns was sequentially eluted with (i) 50 mM-sodium citrate (pH 5.0)/0.5% (v/v) NP40, (ii) 50 mM-Tris/HCl (pH 7.4)/0.5% (v/v) NP40 and (iii) 100 mM-acetic acid/0.15 M-NaCl. Each eluate was immunoprecipitated with JS 8 and control medium and immunoprecipitates analysed by SDS/polyacrylamide-gel electrophoresis.

carbohydrate attachment to glycoproteins (reviewed by Yamada & Olden, 1978). Monoclonal antibody JS 8 immunoprecipitated a protein from tunicamycin-treated HD3 cells of M_r 82000 compared with the 90000- and 95000- M_r proteins found in untreated cells (Fig. 4). This indicated that the chicken transferrin receptor contains N-linked carbohydrate modifications and that JS 8 probably recognizes the peptide portion of the molecule. The M_r of the non-glycosylated form at M_r 82000 is slightly greater than that reported for the human transferrin receptor in Molt-4 cells (78000; Schneider *et al.*, 1982), and indicates that the differences in M_r of the glycosylated forms of the proteins (comparing data from Schneider *et al.*, 1982 and our own unpublished data with Fig. 1) is not due solely to differences in carbohydrate.

Subcellular localization of chicken transferrin receptor

If the 95000- M_r protein recognized by JS 8 was indeed the transferrin receptor, then, by analogy with the mammalian receptor, it would be expected to be primarily located in intracellular transport vesicles, along with conalbumin, with only 10–20% of the receptor being on the cell surface (Ciechanover *et al.*, 1983). We investigated this by indirect immunofluorescence, co-staining HD3 cells with affinity-purified rabbit anti-conalbumin immunoglobulin and JS 8. Fig. 5(d) shows that, as expected, anti-conalbumin antibody stains numerous intracellular vesicles in permeabilized HD3 cells, with relatively little staining of the cell surface. This

probably reflects rapid internalization of receptor-bound complexes, similar to that seen with human cells (Ciechanover *et al.*, 1983). In the same cells, JS 8 also strongly stains these intracellular vesicles, coinciding with the anti-conalbumin staining (Figs. 5c and 5d). In addition, however, there is also strong labelling of the cell membrane. As a control to show that cell-surface glycoproteins in general are not internalized into these vesicles, the cells were stained for an unrelated surface antigen found on chicken erythroblasts using monoclonal antibody JS 5. This antibody showed strong labelling of the cell membrane, no staining of the numerous vesicles recognized by anti-conalbumin antibodies, but weak staining of an intracellular region, G (Figs. 5e and 5f). This region is also a site of staining with anti-conalbumin antibody (Fig. 5f). To investigate whether this region was the Golgi complex, we co-stained permeabilized HD3 cells with Rhodamine-labelled wheat-germ agglutinin and JS 5 or JS 8, followed by fluorescein-labelled goat anti-mouse immunoglobulin, since wheat-germ agglutinin has been reported to preferentially label the Golgi complex in such experiments (Virtanen *et al.*, 1980; Wills *et al.*, 1984). Figs. 5(g)–5(j) show that this region stained by both JS 5 and JS 8 is probably the Golgi complex. This reaction is most likely staining of newly synthesized protein during transit through the Golgi to the plasma membrane (Ciechanover *et al.*, 1983).

Taken together, these data suggest that the subcellular location of the protein recognized by JS 8 is essentially

Fig. 3. Pulse-chase labelling of transferrin receptor in HD3 cells

Cells (10^7) were pulse-labelled for 5 min with L-[35 S]-methionine and chased for up to 2 h in excess unlabelled methionine (see the Materials and methods section). Aliquots (1.5×10^6 cells) were taken after the pulse period (P) and at the chase times indicated, lysates prepared, and immunoprecipitates were analysed by SDS/polyacrylamide-gel electrophoresis. C is a control immunoprecipitation of the 120 min chase sample as is representative of all control immunoprecipitations.

the same as internalized conalbumin but with additional expression of the chicken transferrin receptor on the cell membrane. Interestingly, the internalized conalbumin also cycled into the Golgi apparatus, probably en route back to the cell membrane, as has been shown to occur with mammalian transferrin (reviewed by Ciechanover *et al.*, 1983).

DISCUSSION

Our interest is in the events controlling erythropoiesis in the chicken; therefore in view of the importance of the transferrin receptor in mammalian erythropoiesis, we decided that it was important to be able to identify and analyse the chicken transferrin receptor. Preliminary experiments using antibodies against the human transferrin receptor indicated that we could not identify the chicken counterpart by relying on cross-reactivities with available antisera. However, using monoclonal antibody JS 8, we identified an antigen which bore similarities to the mammalian transferrin receptor. This protein had an M_r of approximately the same value as that of the mammalian transferrin receptor (Schneider *et al.*, 1982) and could be shown to act as a receptor for chicken transferrin, since it was capable of binding to an affinity matrix of immobilized conalbumin and was eluted under

Fig. 4. Avian transferrin receptor is modified with *N*-linked carbohydrate chains

HD3 cells (5×10^6) were incubated in the presence or absence of tunicamycin ($5 \mu\text{g/ml}$) for 2 h before labelling with L-[35 S]-methionine, also in the presence or absence of the inhibitor as necessary. Total cell lysates were prepared, immunoprecipitated with JS 8 and analysed by SDS/polyacrylamide-gel electrophoresis.

conditions that would be expected to specifically elute the mammalian receptor from similar transferrin affinity columns.

Further analysis showed that the chicken transferrin receptor had certain structural similarities to the mammalian receptor. As with the mammalian receptor, it was synthesized as a protein of approx. M_r 5000 smaller than the mature $95000 M_r$ form found on the cell surface. It also existed, albeit only partially, as disulphide-linked dimer on the cell surface. Analysis of the mammalian receptor showed it all to be disulphide-linked as a homodimer; however, the chick cells we analysed require added reducing agents in their normal growth medium, similar to mammalian erythroid precursors (Iscoe *et al.*, 1980) and therefore it is possible that the chicken receptor is partially reduced by the reducing agents during extraction. Alternatively it may in fact only partially exist as a disulphide-linked dimer. Also, similarly to mammalian transferrin receptor, the chicken receptor is modified with *N*-linked glycosylation. The M_r of the protein synthesized in tunicamycin-treated HD3 cells was 82000, slightly larger than that reported for the mammalian receptor in Molt-4 cells (78000; Schneider *et al.*, 1982). This probably reflects the slightly higher M_r of the chicken receptor (95000 rather than 90000 for the human receptor; Schneider *et al.*, 1982) and suggests differences

Fig. 5. Subcellular localization of avian transferrin receptor analysed by indirect immunofluorescence

Fixed, permeabilized HD3 cells were stained with various primary and secondary antibody mixtures as described in the Materials and methods section. (a, b) Negative controls. Cells were co-labelled with a mixture of normal rabbit serum and NS₁ spent-tissue-culture supernatant, followed with fluorescein-labelled goat anti-mouse serum and Rhodamine-labelled swine anti-rabbit serum (see the Materials and methods section for details of dilutions). The same field was viewed for fluorescein (a) or Rhodamine (b) fluorescence. (c, d) Cells co-labelled with a mixture of affinity-purified rabbit anti-conalbumin immunoglobulin and JS 8, detected with the same fluorescent second antibodies as used for control cells. The same field was viewed for fluorescein (c, detecting JS 8) or Rhodamine (d, detecting conalbumin) fluorescence. Note the co-staining of intracellular vesicles and membrane staining by JS 8. (e, f) As for c and d, except that monoclonal antibody JS 5 was used in place of JS 8. Note the lack of intracellular vesicle staining by JS 5. (g, h, i, j) Co-labelling with monoclonal antibodies JS 8 or JS 5 (detected with fluorescein-labelled goat anti-mouse) and Rhodamine-labelled wheat-germ agglutinin (used at 0.025 mg/ml). (g, h) The same field showing (g) JS 8 staining and (h) Rhodamine-wheat-germ agglutinin staining. (i, j) Comparative labelling with monoclonal antibody JS 5 (i) and Rhodamine WGA (j). Note the labelling of the Golgi region (G) with wheat-germ agglutinin and the co-staining of this region with both monoclonal antibodies. The bar (bottom right) represents 10 μ m.

between the molecules. Analysis of the human transferrin receptor cDNA sequences has suggested that the protein is unusual, with the C-terminus of the protein extracellular and the N-terminus intracellular (Schneider *et al.*, 1984). It will be of interest to determine whether the chicken receptor also shows this orientation.

Fluorescent staining with JS 8 was used to investigate the subcellular distribution of transferrin receptors in permeabilized HD3 cells. This had the advantage that it was possible to co-stain with anti-conalbumin antibodies, and the results showed that the chicken transferrin receptor co-localizes with conalbumin in intracellular vesicles (Fig. 5). The additional staining of the cell surface with JS 8 not seen with the anti-conalbumin antibody indicates that unoccupied receptor is present on the cell surface. Studies using Rhodamine-labelled wheat-germ agglutinin, which has been reported to stain preferentially the Golgi apparatus in such fluorescent experiments (Virtanen *et al.*, 1980; Wills *et al.*, 1984), suggested that internalized conalbumin is also in the Golgi complex in HD3 cells (Fig. 5). These data are consistent with recycling of transferrin receptors with bound apo-transferrin through the Golgi complex en route to the plasma membrane (see Ciechanover *et al.*, 1983) and indicate that this process is similar in mammalian and avian cells.

Here we have shown, therefore, that the transferrin receptor present on the surface of chick cells has biochemical properties similar to those of the mammalian transferrin receptor. This is a particularly important finding, since recently two reports have described a transferrin-binding protein from embryonic-chick reticulocytes and neural cells with an M_r of 58000 (Azari & Keung, 1984; Markelonis *et al.*, 1985). The only proteins of approximately this molecular weight seen eluted from conalbumin-Sepharose 4B columns by us were also eluted from bovine serum albumin control columns. The 58000- M_r transferrin receptor was, however, isolated from embryonic cells, whereas the 95000- M_r protein recognized by JS 8 is found on erythroblasts and reticulocytes on normal bone-marrow cells from hatched chickens. Therefore it is possible that the 58000- M_r transferrin receptor represents an embryonic iron-binding protein of some as-yet-undetermined function, whereas JS 8 recognizes a transferrin receptor similar to that found on mammalian cells.

Finally, although our biochemical evidence shows that the chicken transferrin receptor resembles the mammalian receptor, immunological analyses suggest dissimilarities. Sequence analysis between the murine and human receptors (Schneider *et al.*, 1984; Stearne *et al.*, 1985) clearly show a high degree of conservation, although this is mainly localized in discrete domains. It will therefore be of interest to determine to what degree chicken and mammalian transferrin receptors themselves are homologous. This will most easily be done by cloning and sequencing the chicken receptor cDNA and comparing the sequences with those of mammalian transferrin-receptor cDNA species.

We thank Dr. H. Beug for his helpful suggestions and advice and Dr. J. Wyke and Dr. R. Watson for critically reading the

manuscript. Thanks are also due to Miss A. Sterlini for typing the manuscript.

REFERENCES

- Adkins, B., Beug, H. & Graf, T. (1985) *J. Cell. Physiol.* **123**, 269–276
- Aisen, P. & Listowski, I. (1980) *Annu. Rev. Biochem.* **49**, 357–393
- Azari, P. & Keung, W.-M. (1984) *J. Protein Chem.* **3**, 19–332
- Beug, H., von Kirchbach, A., Doederlein, G., Conscience, J.-F. & Graf, T. (1979) *Cell* **18**, 375–380
- Beug, H., Doederlein, G., Freudenstein, C. & Graf, T. (1982) *J. Cell. Physiol.* **115**, 295–309
- Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88
- Ciechanover, A., Schwartz, A. L. & Lodish, H. (1983) *J. Cell. Biochem.* **23**, 107–130
- Faulk, W. P., Hsi, B.-L. & Stevens, P. J. (1980) *Lancet* **ii**, 390–392
- Fernandez-Pol, J. A. & Klos, D. J. (1980) *Biochemistry* **19**, 3904–3912
- Hayman, M. J., Beug, H. & Savin, K. (1982) *J. Cell. Biochem.* **18**, 351–362
- Hu, H. Y. Y. & Aisen, P. (1978) *J. Supramol. Struct.* **8**, 349–360
- Hunt, R. C., Ruffin, R. & Yang, Y.-S. (1984) *J. Biol. Chem.* **259**, 9944–9952
- Iacopetta, B. J., Morgan, E. H. & Yeoh, G. C. T. (1982) *Biochim. Biophys. Acta* **687**, 204–210
- Iscove, N. N., Guilbert, L. J. & Weyman, C. (1980) *Expt. Cell Res.* **126**, 121–126
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Larrick, J. W. & Cresswell, P. (1979a) *J. Supramol. Struct.* **11**, 579–586
- Larrick, J. W. & Cresswell, P. (1979b) *Biochim. Biophys. Acta* **583**, 483–490
- Markelonis, G. J., Oh, T. H., Park, L. P., Cha, C. Y., Sofia, C. A., Kim, J. W. & Azari, P. (1985) *J. Cell Biol.* **100**, 8–17
- Newman, R., Schneider, C., Sutherland, R., Vodinelich, L. & Greaves, M. (1982) *Trends Biochem. Sci.* **7**, 397–400
- Omary, M. B. & Trowbridge, I. S. (1981) *J. Biol. Chem.* **256**, 12888–12892
- Seligman, P. A., Schleicher, R. B. & Allen, R. H. (1979) *J. Biol. Chem.* **254**, 9943–9946
- Schmidt, J. A., Beug, H. & Hayman, M. J. (1985a) *EMBO J.* **4**, 105–112
- Schmidt, J. A., Marshall, J., Hayman, M. J., Doderlein, G. & Beug, H. (1985b) *Leukaemia Res.*, in the press
- Schneider, C., Sutherland, R., Newman, R. A. & Greaves, M. F. (1982) *J. Biol. Chem.* **257**, 8516–8522
- Schneider, C., Kurkinen, M. & Greaves, M. (1983) *EMBO J.* **2**, 2259–2263
- Schneider, C., Owen, M. J., Banville, D. & Williams, J. G. (1984) *Nature (London)* **311**, 675–678
- Stearne, P. A., Pietersz, G. A. & Goding, J. W. (1985) *J. Immunol.* **134**, 3474–3479
- Trowbridge, I. S. & Lopez, F. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 1175–1179
- Trowbridge, I. S. & Omary, M. B. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3039–3043
- Virtanen, I., Ekblom, P. & Laurila, P. (1980) *J. Cell Biol.* **85**, 429–434
- Wills, J. W., Srinivas, R. V. & Hunter, E. (1984) *J. Cell Biol.* **99**, 2011–2023
- Yamada, K. M. & Olden, L. (1978) *Nature (London)* **275**, 179–184